

## Biochemical Characterization Of Mapmodulin, a Protein That Binds Microtubule-associated Proteins\*

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Mapmodulin is a 31-kDa protein that stimulates the microtubule- and dynein-dependent localization of Golgi complexes in semi-intact Chinese hamster ovary cells. We have shown previously that it binds the microtubule binding domains of the microtubule-associated proteins, MAP2, MAP4, and tau. We also showed that mapmodulin is identical to a protein named PHAPI (Vaesen, M., Barnikol-Watanabe, S., Götz, H., Awni, L.A., Cole, T., Zimmermann, B., Kratzin, H.D. and Hilschmann, N. (1994) *Biol. Chem. Hoppe-Seyler* 375, 113-126). We report here that mapmodulin is a phosphoprotein that is predominantly cytosolic but is also found peripherally associated with endoplasmic reticulum and Golgi membranes in mammalian cells. The protein occurs as a trimer in cytosol, and phosphorylation is required for its microtubule-associated protein-binding activity. Heat treatment of nonphosphorylated mapmodulin can render it competent for binding to microtubule-associated proteins, suggesting that phosphorylation induces a conformational change in mapmodulin. Finally, despite identity in polypeptide sequence with a protein reported to act as an inhibitor of protein phosphatase 2A, native mapmodulin was not able to inhibit protein phosphatase 2A in Chinese hamster ovary cell cytosol.

When isolated Golgi complexes are mixed with broken CHO<sup>1</sup> cells, they enter the cytoplasm and accumulate at the centrosome. This "Golgi capture" process requires intact microtubules, cytoplasmic dynein, ATP hydrolysis, as well as cytosolic and membrane-associated proteins (1). We have recently described the purification of a 31-kDa protein, named mapmodulin, that appears to be essential for Golgi capture (2).

We have shown that mapmodulin binds MAP4, MAP2, and tau (2). It binds free MAPs in strong preference to microtubule-bound MAPs and does so via the microtubule-binding domain of the MAP. Binding to each of these MAPs requires the carboxyl-terminal portion of mapmodulin and decreases the rate with which MAPs can subsequently re-bind to microtubules. We have proposed that mapmodulin represents a novel class of proteins that functions to displace MAPs transiently from microtubules, thereby permitting organelle translocation (2).

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<sup>1</sup> The abbreviations used are: CHO, Chinese hamster ovary; PHAPI, putative HLA-associated protein-I; HLA, human major histocompatibility antigen; ER, endoplasmic reticulum; MAP, microtubule-associated protein; PIPES, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FPLC, fast protein liquid chromatography; GST, glutathione S-transferase.

Peptide sequence analysis of mapmodulin revealed complete identity with "putative HLA-associated protein-I" (PHAPI; Ref. 3). PHAPI was purified from lymphoblastoid cell cytosol based on its ability to bind to a peptide corresponding to the cytoplasmic domain of the human HLA class II DR2 $\alpha$ -chain. Despite the basic nature of the  $\alpha$ -chain peptide employed, control experiments showed that basic residues were not sufficient for PHAPI binding (3). Preliminary immunoelectron microscopy detected the protein over all cellular compartments, including the cytoplasm, membranes, and nucleus (3). The same protein was also purified independently as LANP (4), pp32 (5), and I<sub>1</sub><sup>PP2A</sup> (6, 7). I<sub>1</sub><sup>PP2A</sup> was reported to specifically inhibit protein phosphatase 2A in *in vitro* assays (6, 7).

In this paper, we further characterize mapmodulin/PHAPI and show that it is an abundant cytosolic phosphoprotein, partially associated with ER and Golgi membranes. The predominant form of mapmodulin in cells is phosphorylated; phospho-mapmodulin binds to the MAP4 microtubule-binding domain with higher affinity than the dephosphorylated form, suggesting that this posttranslational modification plays a role in regulating MAP binding.

Sequence alignments revealed that mapmodulin shares limited structural homology with CLIP-170, a putative endosome-microtubule linker protein (8, 9). Despite this sequence similarity, mapmodulin does not serve as a linker between the Golgi complex and microtubules. Finally, neither native CHO mapmodulin nor bacterially expressed, His-tagged mapmodulin inhibit protein phosphatase 2A under physiological conditions, in contrast to reports from another laboratory for the protein purified as I<sub>1</sub><sup>PP2A</sup>.

### EXPERIMENTAL PROCEDURES

**Materials**—Antibodies, CHO mapmodulin, recombinant, His-tagged mapmodulin, and Golgi membranes were prepared as described previously (2).

**In Vitro Golgi Binding Assay**—Purified bovine tubulin (10 mg/ml) was polymerized by incubation with 1 mM GTP and 20  $\mu$ M taxol for 15 min at 37 °C. After ultracentrifugation, pellets were resuspended in PE buffer (85 mM K-PIPES, pH 7.0, 1 mM EGTA, 1.5 mM MgSO<sub>4</sub>, 10  $\mu$ M taxol) to a final concentration of 1 mg/ml. In a standard reaction, 6  $\mu$ g of KCl-stripped Golgi membranes were incubated with 20  $\mu$ g of microtubules in PE buffer for 30 min at 37 °C in the presence or absence of 100  $\mu$ g of cytosol. The reaction mix (50  $\mu$ l) was layered over 40% sucrose in PE buffer and spun at 35000 rpm for 30 min at 25 °C in a TLA-100 rotor (Beckman Instruments, Palo Alto, California). Pellets were subjected to an *N*-acetylglucosamine transferase I enzymatic assay (2).

**Cell Fractionation and Immunoblot Analysis**—CHO cells were homogenized in SEAT buffer (250 mM sucrose, 10 mM triethanolamine, 10 mM acetic acid, 1 mM EDTA, pH 7.4) by 5 passages through a 22-gauge needle. Nuclei and debris were pelleted by centrifugation at 4000 rpm for 5 min in an Eppendorf centrifuge. The postnuclear supernatant was centrifuged at 60,000 rpm for 30 min at 4 °C in a Beckman TLA-100 rotor, and aliquots of the pellet and supernatant fractions were analyzed by SDS-PAGE followed by immunoblotting.

**Immunofluorescence**—Immunofluorescence was carried out essentially as described by Warren *et al.* (10). The cells were permeabilized using 0.5% saponin or 0.2% Triton X-100 in PBS for 5 min and then

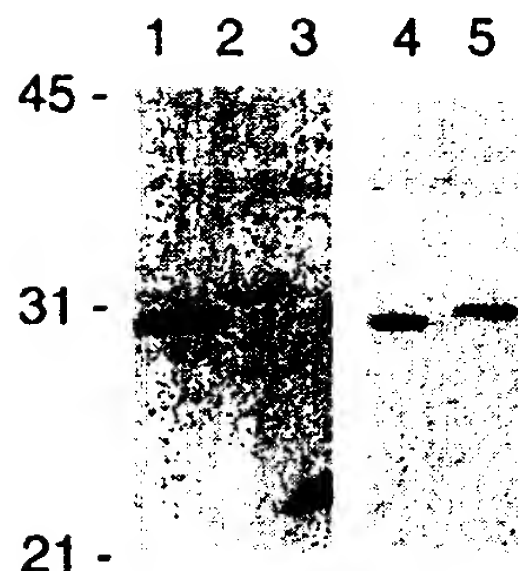


FIG. 1. SDS-PAGE (12.5%) and Coomassie Blue staining (lanes 1–3) or immunoblot analysis (lanes 4 and 5) of purified mapmodulin from CHO cells (lane 1, 2  $\mu$ g) and recombinant, His-tagged mapmodulin/PHAPI or mapmodulin $\Delta$ C (lanes 2 and 3, 2  $\mu$ g). CHO cytosol (lane 4, 250  $\mu$ g) or recombinant mapmodulin/PHAPI (lane 2, 30 ng) were detected with either anti-native mapmodulin (lane 4) or anti-recombinant mapmodulin (lane 5). Mobility of molecular weight markers is shown at left (in kDa).

incubated with anti-mapmodulin affinity-purified antibodies for 1 h at 22 °C. After washing, the cells were incubated with Texas Red-conjugated anti-rabbit IgG diluted 1:500. Slides were viewed using a Zeiss Axiophot microscope equipped with a Texas red filter (Omega Optical Inc., Brattleboro, VT) with a  $\times$  100 Plan Neofluor, 1.3 oil immersion objective; images were recorded on Fuji 1600 film.

**MAP4 Binding Assay**—The solid phase binding immunoassays were performed exactly as described (2).

**Phosphatase Assay**—Phosphatase assays were carried out using the Life Technologies, Inc. protein phosphatase assay system. Reaction mixtures contained assay buffer (20 mM imidazole-HCl, pH 7.4, 0.1%  $\beta$ -mercaptoethanol, 1 mg/ml bovine serum albumin, 0.1 mM EDTA), 20  $\mu$ l of CHO cytosol, 20  $\mu$ l of  $^{32}$ P-labeled phosphorylase  $\alpha$  (in 50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 15 mM caffeine, 1%  $\beta$ -mercaptoethanol), in a final volume of 60  $\mu$ l. Reactions were initiated with the  $^{32}$ P-labeled substrate, and after 10 min at 30 °C, 180  $\mu$ l of 20% trichloroacetic acid was added. After incubating on ice for 20 min, samples were centrifuged at 12,000  $\times$  g for 10 min in a microcentrifuge. The radioactivity in 200  $\mu$ l of the supernatant was then measured in a scintillation counter. The effect of mapmodulin was measured by incubating cytosol with different concentrations of purified mapmodulin, or commercially purchased I1 $^{125}$ A (CalBiochem). The concentration of cytosol used corresponded to the low linear range of phosphatase activity (2.9  $\mu$ g/ml in assay buffer).

**Alkaline Phosphatase Treatment**—CHO cytosol (50  $\mu$ g) was incubated with 1 unit of calf intestine alkaline phosphatase (Boehringer Mannheim) for 1 h at 37 °C as recommended by the manufacturer; the reaction was then terminated by boiling in SDS-PAGE sample buffer.

## RESULTS

Mapmodulin was purified from CHO cytosol on the basis of its ability to stimulate the dynein- and microtubule-dependent localization of Golgi complexes in broken cells (2). As described above, mapmodulin is identical to PHAPI, a protein that was purified from human lymphoblastoid B-cell line cytosol on the basis of its capacity to bind peptides representing the basic, cytosolic tail of HLA class II  $\alpha$ -chain (3).

Mapmodulin (PHAPI) has a predicted mass of 28.4 kDa (3). When the native protein purified from CHO cytosol is resolved by SDS-PAGE under denaturing conditions, the protein has an apparent molecular mass of  $\sim$ 31 kDa (Fig. 1). We have prepared a His-tagged recombinant form of mapmodulin and purified the protein after expression in *Escherichia coli* cells (2) (Fig. 1, lane 2); a His-tagged, carboxyl-terminal truncated form, which lacks the acidic region encoded by residues 164–249, was also generated (2) (lane 3).

Mapmodulin chromatographs as an apparent oligomer upon gel filtration chromatography. When purified CHO cell mapmodulin was fractionated on a Superdex 200 FPLC column at physiological ionic strength (PBS), it eluted just ahead of the 76-kDa marker, suggesting that under physiological condi-

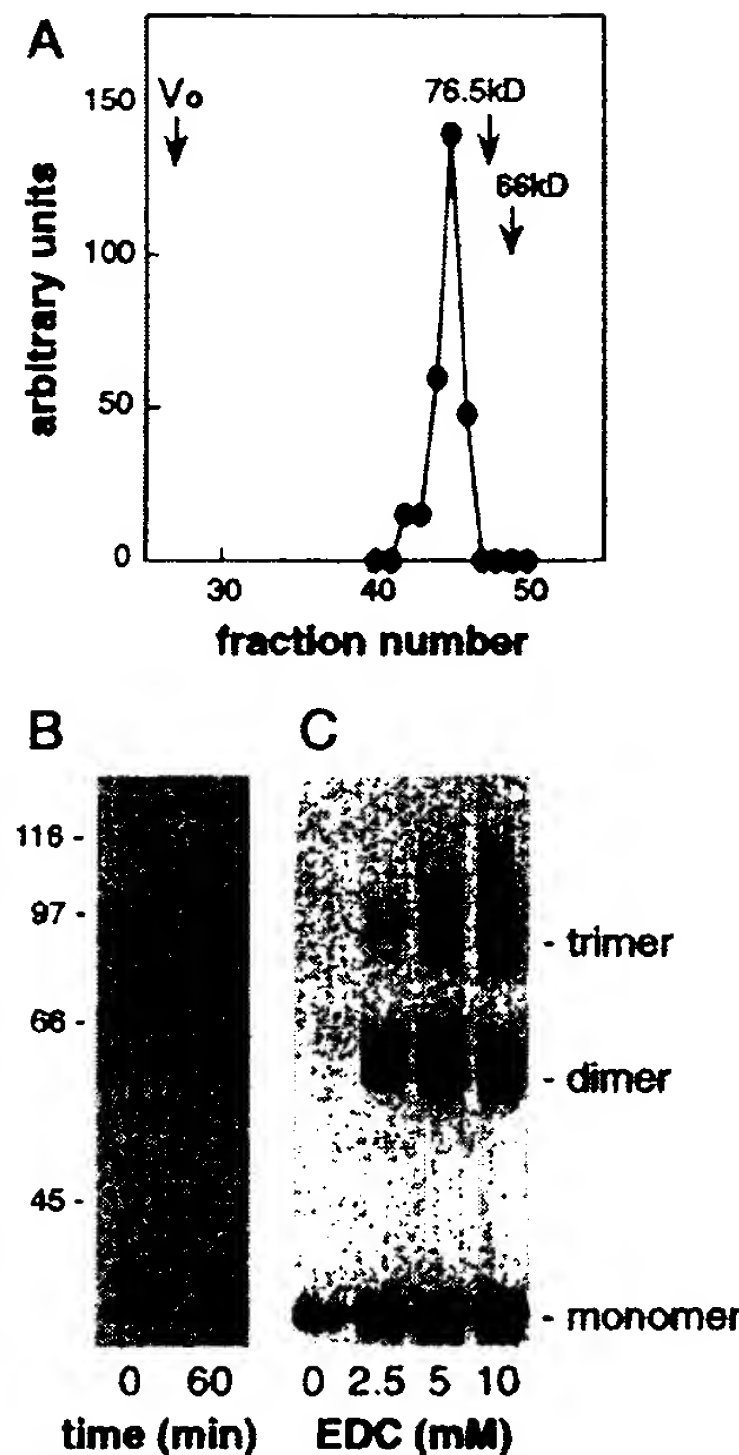


FIG. 2. Mapmodulin appears to be a trimer. A, elution of CHO mapmodulin from Superose 200 FPLC (120-ml column). Mapmodulin (20  $\mu$ g in 1 ml), together with transferrin (76.5 kDa) and bovine serum albumin (66 kDa; 20  $\mu$ g each), was injected on the column and run in PBS buffer. B, CHO cell mapmodulin (500 ng) was incubated with 0.1 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and 5 mM *N*-hydroxysulfosuccinimide for 0 or 60 min at 22 °C. The reaction was terminated by adding 0.36 M  $\beta$ -mercaptoethanol. Samples were resolved on 8% SDS-PAGE and electroblotted, and the cross-linked products were detected with polyclonal anti-mapmodulin IgG. The migration of molecular mass markers is shown at left in kDa. C, recombinant, His-tagged mapmodulin $\Delta$ C (1.5  $\mu$ g) was incubated with the indicated concentrations of EDC supplemented with 5 mM *N*-hydroxysulfosuccinimide for 30 min at 22 °C. Samples were resolved by 10% SDS-PAGE; proteins were detected with polyclonal anti-recombinant mapmodulin antibodies. Please note that mapmodulin $\Delta$ C is smaller than full-length mapmodulin, thus the "trimer" of this protein is not the same size as the mapmodulin trimer shown in panel B.

tions, the native protein may in fact be a trimer (Fig. 2A). Cross-linking experiments carried out with purified, CHO cell mapmodulin (Fig. 2B) or recombinant, His-tagged mapmodulin $\Delta$ C (Fig. 2C) revealed that both forms of the protein form trimers. These data suggest that mapmodulin is likely a trimer in CHO cytosol. In addition, the ability of the carboxyl-terminal truncated form to form apparent trimers indicates that the carboxyl terminus of the protein is not required for mapmodulin homo-oligomerization.

**Mapmodulin Localization**—Polyclonal antibodies were raised against purified, CHO mapmodulin and recombinant, His-tagged mapmodulin. In total CHO cytosol, anti-native mapmodulin recognized a single polypeptide by immunoblot analysis with an apparent mass of  $\sim$ 31 kDa (Fig. 1, lane 4). The CHO cell protein was smaller in mass than the purified, recombinant mapmodulin, which contained additional histidine residues (Fig. 1, lane 5). The polyclonal anti-CHO mapmodulin antibodies were used to localize mapmodulin by indirect immunofluorescence of CHO cells. Using mild conditions for permeabilization (0.5% saponin), interphase cells exhibited an

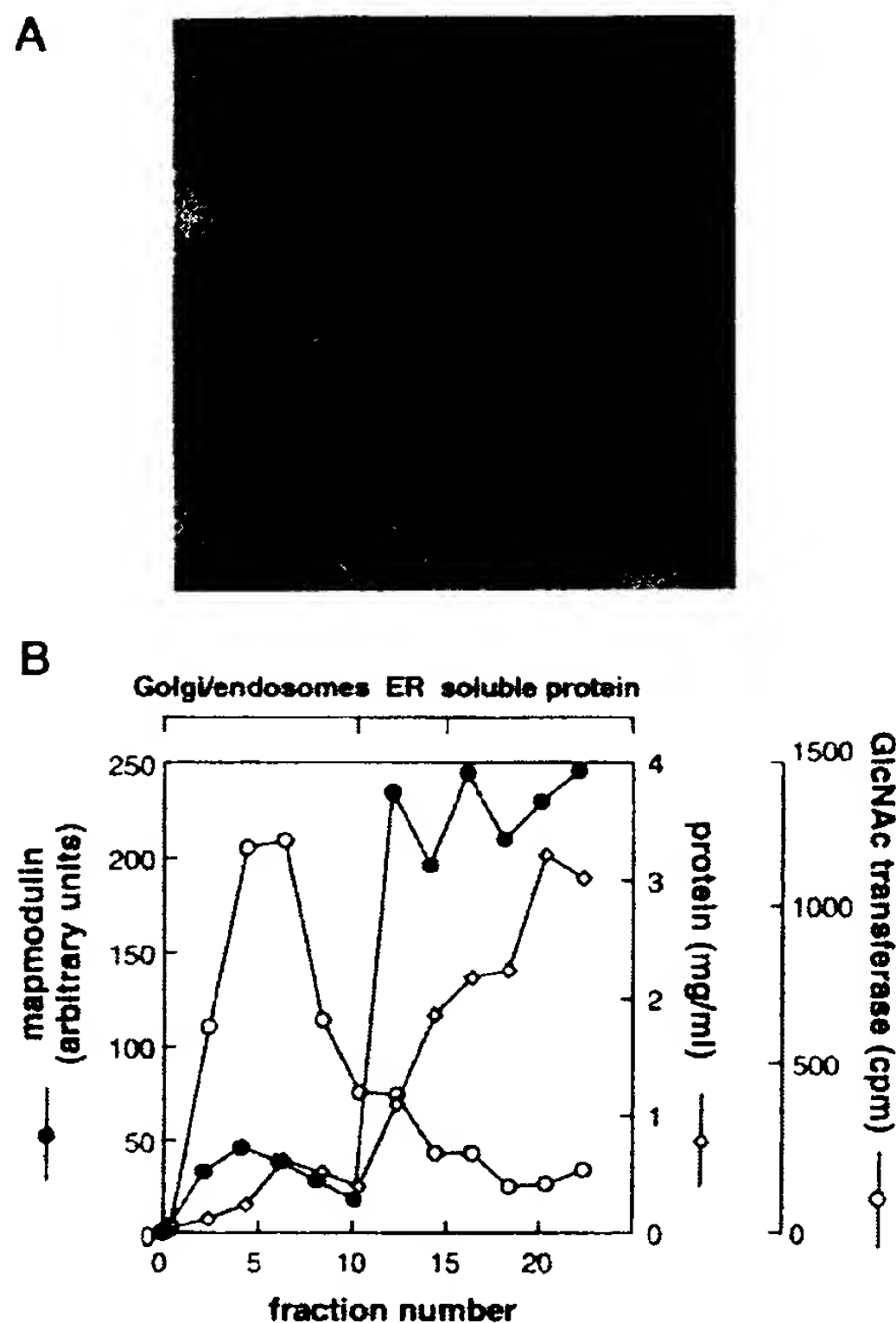


FIG. 3. Cellular distribution of mapmodulin. A, immunofluorescence of CHO cells. Cells were permeabilized with 0.2% Triton X-100; mapmodulin was visualized using affinity-purified polyclonal antibodies and Texas Red-conjugated anti-rabbit secondary antibodies (1:500). Anti-native mapmodulin and anti-recombinant mapmodulin yielded the same labeling pattern. B, sucrose gradient flotation of a CHO cell postnuclear supernatant. The sample was adjusted to 1.4 M sucrose and overlaid with 1.2 and 0.8 M sucrose in 10 mM Tris/HCl, pH 7.4. Centrifugation was carried out at 36,000 rpm for 2.5 h in a Beckman SW41 rotor. Fractions were collected from the top and assayed for the indicated components. Mapmodulin was determined by immunoblot.

overall diffuse staining, as would be expected from a cytosolic protein. More rigorous detergent pre-extraction (0.2% Triton X-100) led to a clearer image of uniform labeling of what appeared to be both ER and Golgi membranes, suggesting that mapmodulin occurs in both soluble and membrane-bound forms (Fig. 3A). With longer times of detergent extraction, nuclear staining is also observed. These findings were consistent with previous preliminary immunoelectron microscopic localization of PHAPI in lymphoblastoid cells (3).

To verify the membrane interaction of mapmodulin, we subjected a CHO cell postnuclear supernatant to flotation in a sucrose gradient. In these gradients, membrane-bound organelles float upward and can be readily resolved from cytosolic proteins. As shown in Fig. 3B, the bulk of a Golgi marker, *N*-acetylglucosamine transferase I (○, fractions 3–9), was well separated from the bulk of the protein that remained at the bottom of the gradient (◇, fractions 15–22). Endosomes and lysosomes co-fractionate with Golgi on such gradients; ER membranes float to an intermediate density (not shown). While the majority of mapmodulin fractionated with soluble protein (●), a significant amount was detected in the lighter density, membrane-enriched regions of the gradient. These biochemical data confirm that mapmodulin occurs in both soluble and membrane-associated forms. Quantitation of fractionated CHO cells

revealed that 3–5% of the cellular pool of mapmodulin is membrane-bound. This fraction can be stripped off of membranes with 0.5 M KCl (data not shown).

**Mapmodulin Shows Discrete Homology to CLIP-170**—Fig. 4A presents the sequence of PHAPI (3) and the tryptic peptides we obtained from purified, CHO mapmodulin. The mapmodulin/PHAPI sequence is comprised of a generally basic, Leu/Ile-rich, amino-terminal region and a long, acidic carboxyl-terminal portion; the protein has an overall calculated pI of 3.7. The sequence does not contain consensus nucleotide binding sites and thus is unlikely to represent a molecular motor. Interestingly, we found that mapmodulin shares small segments of similarity with CLIP-170, the putative cytoplasmic linker between endosomes and microtubules (8, 9). Three of the four segments display 35–60% sequence identity and 53–80% similarity without introduction of any gaps or deletions (Fig. 4B). The homologous segments reside within the amino-terminal region of mapmodulin and the carboxyl-terminal domain of CLIP-170, and their order and relative orientation is conserved between the two proteins. The highest level of identity (60%) was found between residues 145–154 of mapmodulin and residues 1313–1322 of CLIP-170, part of the putative organelle-binding domain of CLIP-170 (9). While this homology is intriguing, its true significance is at present, entirely unclear.

**Mapmodulin Does Not Mediate Binding of Golgi to Microtubules**—Given the sequence similarity with CLIP-170, it was of interest to determine whether mapmodulin can link the Golgi to microtubules. For this purpose, we used an assay established by Kreis and co-workers (8, 11) to detect CLIP-170-mediated, endosome-microtubule interactions. Taxol-stabilized bovine microtubules were incubated with KCl-treated Golgi membranes in the presence or absence of added CHO cytosol at 37 °C for 30 min. Reactions were then centrifuged through a sucrose cushion to separate free Golgi membranes from those that may have become microtubule-bound. The extent of Golgi binding was then quantified by measuring the level of Golgi-specific enzyme, *N*-acetylglucosamine transferase I, in the microtubule pellet.

As shown in Table I, cytosol increased the association of KCl-treated Golgi with microtubules at least 7-fold. In contrast, CHO cell mapmodulin alone did not stimulate the association of KCl-treated Golgi with microtubules by more than a few percent. We estimate CHO cytosol to contain ~50 ng of mapmodulin/100 µg, comparable with the amount tested in these experiments (64 ng). Thus, mapmodulin does not mediate the binding of Golgi complexes to microtubules under these experimental conditions, when added at concentrations similar to those found in cytosol. This feature distinguishes mapmodulin functionally from CLIP-170, which has been shown to mediate endosome-microtubule association under very similar conditions (8, 11).

**Mapmodulin Is a Phosphoprotein**—Two-dimensional gel analyses of mapmodulin in CHO cytosol revealed that the protein is phosphorylated. As shown in Fig. 5A, antibodies raised against native CHO mapmodulin recognized one major and one minor spot on a two-dimensional gel immunoblot. When cytosol was treated with alkaline phosphatase, the bulk of the immunoreactivity shifted to the position of the slightly smaller, less acidic spot (panel B). Thus, the major form of mapmodulin is phosphorylated in CHO cytosol.

Antibodies raised against the recombinant protein were much less effective in recognizing the phosphorylated form of mapmodulin. Only after phosphatase treatment of CHO cytosol did the antibody yield a significant reaction product (Fig. 5, C and D). It is very likely that the *E. coli* cells used for expression purposes lack the kinases responsible for mapmodulin phos-



## A

MEMGRRIHLELRNRTPSDVKELVLDNSRSNEGKLEGLTDEFEELEFLSTI  
 NVGLTSIANLPKLNKLLKLELSDNRVSGGLEVLAEKCPNLTHLNLSGNKI  
 KDLSTIEPLKKLENLKSLEDFNCEVTNLNDYRENVFKLLPOLTYLDGYDR  
 ↓  
 DDKEAPDSDAEGYVEGLDDEEEDEDEEEYDEDAQVVEDEDEDEEEEGEE  
 EDVSGEEEEDEEGYNDGEVDDEEDEEELGEEERGQKRKREPEDEGEDDD

## B

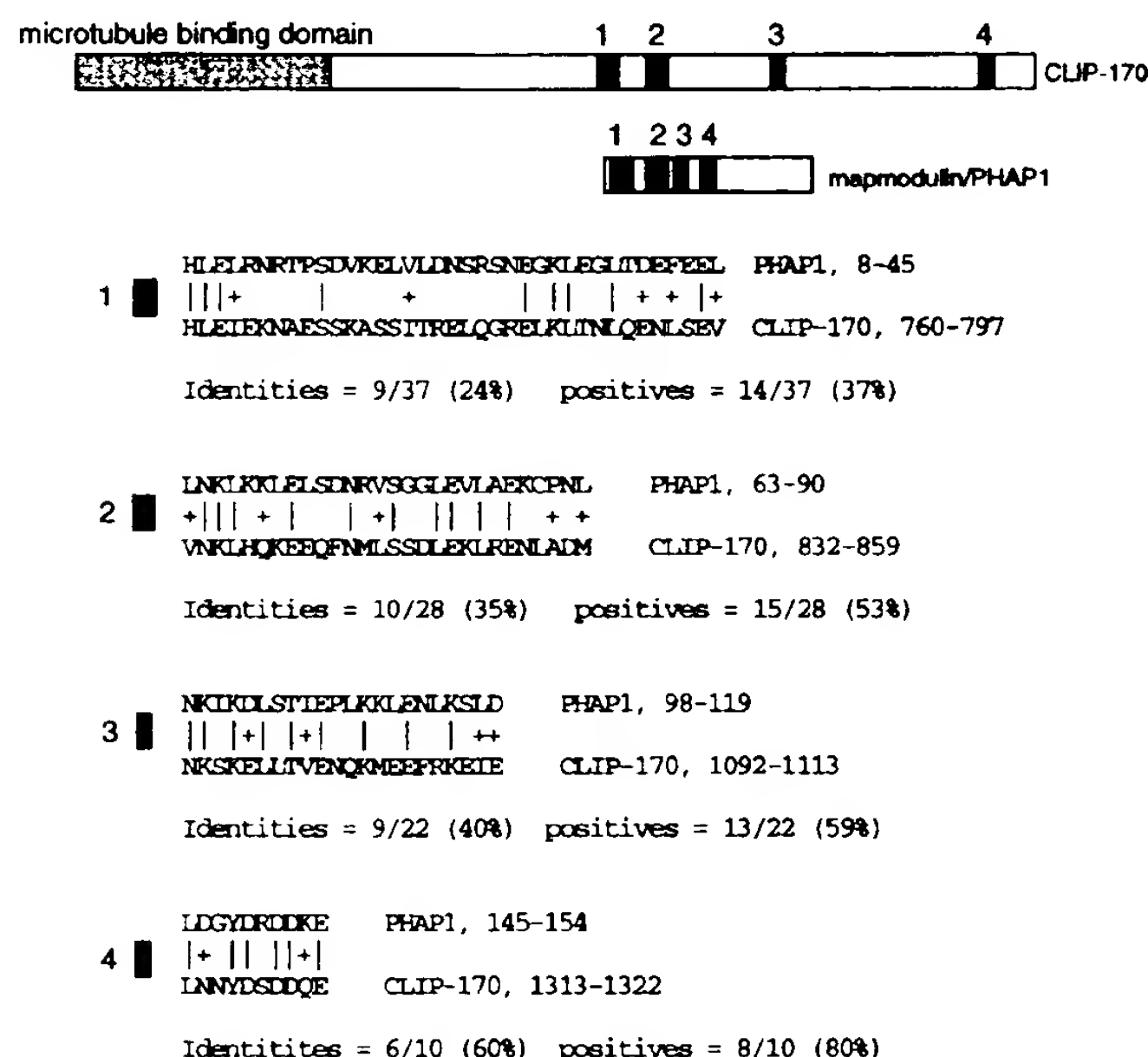


FIG. 4. A, human PHAP1 sequence (from Ref. 3). Underlined are the tryptic peptide sequences we obtained from CHO mapmodulin; for the second peptide, we obtained the sequence LLPQLSYLDGYDREDQEAP rather than that shown. The arrow indicates the carboxyl terminus of mapmodulinΔC. B, regions of homology between mapmodulin/PHAP1 and CLIP-170 (numbered 1-4). A homology search was performed using Blast program in conjunction with the PIR data base; P value of 0.93 was obtained when full-length CLIP170 and mapmodulinΔC were compared. Vertical lines (|) indicate identity and (+) symbols indicate similarity. No gaps or insertions are shown, which would increase the level of similarity.

TABLE I  
 Mapmodulin does not link the Golgi to microtubules

KCl-stripped Golgi complexes (6 μg) were incubated with 20 μg of taxol-stabilized microtubules and CHO cytosol (100 μg) or purified mapmodulin for 30 min at 37 °C. The reaction mix (50 μl) was then layered onto a 40% sucrose cushion and spun at 35,000 rpm for 30 min at 25 °C in a TLA-100 rotor (Beckman). Resulting pellets were subjected to a GlcNAc transferase I assay to monitor Golgi association with microtubules.

	Pelleted	Percent full cytosol
	cpm	
KCl-Golgi + microtubules	461	0
KCl-Golgi + CHO cytosol	3251	100
KCl-Golgi + mapmodulin (64 ng)	667	7.4

phorylation. These experiments show that mapmodulin appears primarily as a phosphoprotein in CHO cytosol.

It should be noted that a smaller (~28 kDa), and more basic (pI = 4.4), immunoreactive spot was occasionally detected in some cytosol preparations. This form was only recognized by anti-recombinant mapmodulin antibodies. Since distinct splice variants of PHAP1 have appeared in the data base, this spot may represent the product of a distinctly spliced mRNA.

**Phosphorylation Is Needed for Mapmodulin-MAP4 Interaction**—Since our previous MAP binding experiments were carried out with native, phosphorylated CHO mapmodulin (2), we were interested in determining the potential importance of

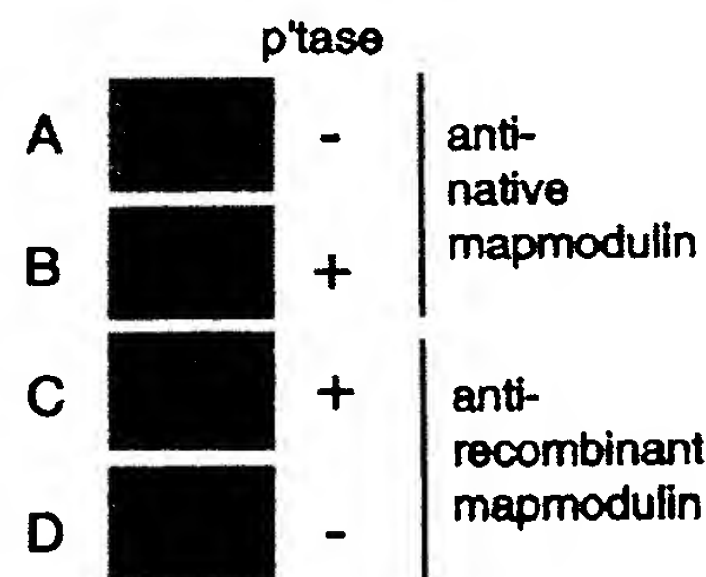


FIG. 5. Two-dimensional gel electrophoresis reveals a phosphorylated form of mapmodulin in CHO cytosol. CHO cytosol, treated with (B and C) or without (A and D) alkaline phosphatase (p'tase), was analyzed by two-dimensional gel electrophoresis. Gels were transferred to nitrocellulose, and blots were reacted with either anti-native mapmodulin (A and B) or anti-recombinant mapmodulin (C and D) polyclonal sera as indicated at right.

mapmodulin phosphorylation in terms of MAP binding. For this purpose, we used a solid phase immunoassay to compare the binding of phospho- and dephospho-mapmodulin to the microtubule binding, carboxyl-terminal domain of MAP4 (12). As shown in Fig. 6, nonphosphorylated, recombinant mapmodulin displayed only barely detectable binding to the carboxyl-terminal domain of MAP4, relative to that observed with

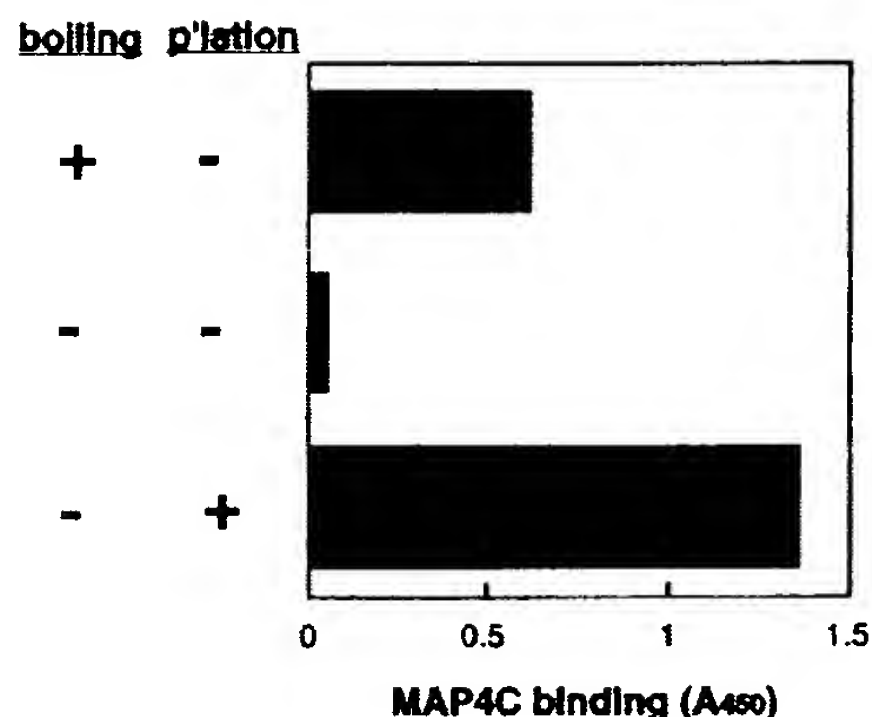


FIG. 6. Phosphorylation is required for binding of mapmodulin to the carboxyl terminus of MAP4. Microtiter wells were coated with MAP4-carboxyl-terminal domain GST-fusion protein. After blocking the wells, either CHO-purified mapmodulin, recombinant mapmodulin, or boiled recombinant mapmodulin was added (each at  $0.23 \mu\text{M}$ ). Bound protein was detected using polyclonal anti-mapmodulin antibodies.

the native form.

When the same preparation of nonphosphorylated mapmodulin was boiled for 10 min, its MAP4 binding capacity was dramatically increased. These results suggest that mapmodulin phosphorylation allows this protein to assume a binding-competent conformation. Unfolding of the nonphosphorylated form of the protein by heat treatment appears to expose its MAP4 binding domain. The fact that a polyclonal antibody raised against the nonphosphorylated form of the protein only poorly recognizes the phosphorylated form is consistent with significant conformational differences between the two forms. Additional experiments will be needed to determine the nature of this conformational transformation.

Direct dephosphorylation of phospho-mapmodulin would be predicted to eliminate its ability to bind MAPs. However, phosphatase treatment was not sufficient to block the MAP-binding capacity of mapmodulin (not shown). It is very possible that phosphatase treatment does not fully dephosphorylate mapmodulin; all we have shown is that at least partial dephosphorylation uncovers an epitope recognized by anti-dephospho-mapmodulin.

Finally, it should be noted that heat treatment of carboxyl-terminal truncated mapmodulin $\Delta\text{C}$  did not generate a molecule that could bind MAP4 (data not shown), reconfirming our previous conclusion that the acidic carboxyl terminus of mapmodulin is essential for MAP4 binding (2).

**Mapmodulin Is Not a Phosphatase Inhibitor**—During the course of our study, Li *et al.* (6, 7) reported the purification of a  $\approx 250$  kDa potent and specific inhibitor of protein phosphatase 2A, which they named  $\text{I}_1^{\text{PP2A}}$ . Sequence analysis of this inhibitor revealed identity with PHAPI. This finding made it incumbent upon us to check whether native mapmodulin possessed phosphatase 2A-inhibitory activity.

Phosphorylase  $\alpha$  was used as a substrate, and CHO cytosol was used as the source of protein phosphatases 1 and 2A. Fig. 7 shows that commercial  $\text{I}_1^{\text{PP2A}}$  was a strong inhibitor of phosphatase 2A activity in CHO cytosol. In contrast, both native and recombinant mapmodulin failed to have any influence on phosphatase activity in this assay. This result was obtained with numerous cytosol and mapmodulin preparations. In summary, native mapmodulin, as purified by our published procedure, does not display phosphatase 2A inhibitory activity using phosphorylase  $\alpha$  as substrate. It is important to note that unlike  $\text{I}_1^{\text{PP2A}}$ , which has an apparent  $M_r$  of  $\geq 250,000$ , native and

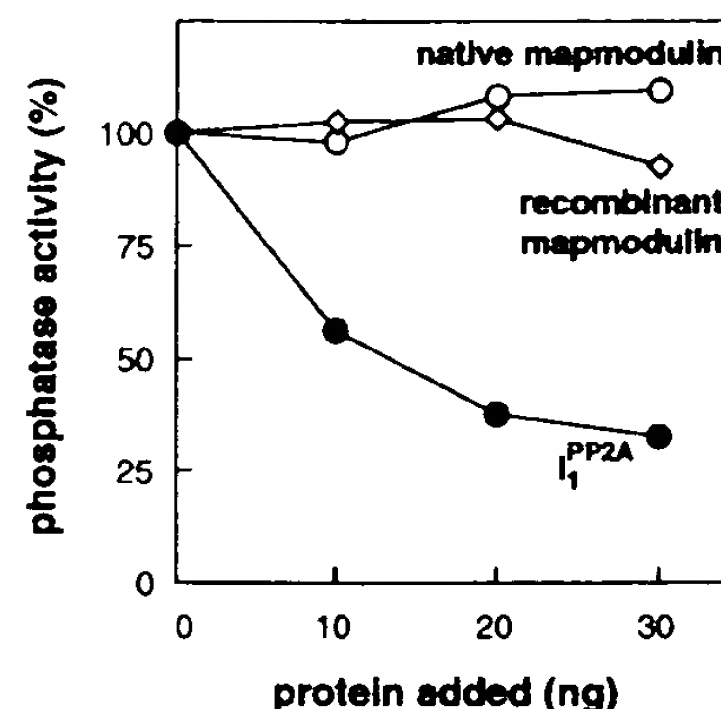


FIG. 7. Native mapmodulin does not inhibit protein phosphatase 2A. The activity of cytosolic phosphatases was measured with  $^{32}\text{P}$ -phosphorylase  $\alpha$  as substrate.

recombinant mapmodulin/PHAPI chromatograph as  $\sim 90$ -kDa species (Fig. 2). This molecular distinction may in part explain the discrepancy between our results and those of Li *et al.* (7).

#### DISCUSSION

Mapmodulin stimulates the microtubule and cytoplasmic dynein-dependent localization of Golgi complexes to the centrosomes of semi-intact Chinese hamster ovary cells (2). In our efforts to understand how this protein stimulates Golgi localization, we found that mapmodulin binds the microtubule-associated proteins, MAP2, MAP4, and tau, via their homologous microtubule-binding domains (2). We proposed that this property may allow mapmodulin to displace MAPs from the path of organelles translocating along microtubules toward the centrosome.

In this paper, we have characterized the cellular localization and posttranslational modification of this novel, MAP-binding protein. By fractionation, the protein is predominantly cytosolic, but at least  $\sim 5\%$  can be shown to float in a sucrose gradient with endosome and Golgi-associated marker proteins. This was consistent with membrane localization determined by immunofluorescence light microscopy. Gel filtration experiments suggested that the cytosolic protein occurs as a trimer. In addition, two-dimensional gel analysis, carried out in conjunction with alkaline phosphatase treatment, confirmed that the major form of mapmodulin in CHO cytosol is phosphorylated.

The primary structure of mapmodulin has two striking features. The carboxyl terminus is highly enriched in acidic residues, and the presence of this domain is required for MAP binding (2). The amino-terminal portion contains a high proportion of leucine residues and a sequence that matches the consensus for a leucine-rich repeat. Leucine-rich repeats are relatively short motifs found in proteins of diverse function and localization. They appear to be able to form amphipathic structures with hydrophobic surfaces capable of interacting with other proteins. Since mapmodulin and carboxyl-terminal truncated mapmodulin apparently form trimers, it seems reasonable to propose that homo-oligomerization is mediated by the leucine-rich repeats.

Here we have shown that mapmodulin phosphorylation is required for MAP binding. Although the kinases responsible for mapmodulin phosphorylation remain to be identified, multiple casein kinase II recognition motifs are present in the amino-terminal domain of the protein. Perhaps the amino-terminal portion interacts strongly with the carboxyl-terminal, MAP-binding domain in the nonphosphorylated state. Phosphorylation might loosen this interaction to permit mapmodulin-MAP interaction. Our finding that boiling of nonphosphorylated

mapmodulin generates a protein that is competent for MAP binding is consistent with this hypothesis.

Recombinant mapmodulin was active in our *in vitro* Golgi localization assay only when reactions were supplemented with additional cytosol (2). It is likely that recombinant mapmodulin is rapidly phosphorylated by the residual cytosol present in our broken cells, converting it to an active conformation.

Sequence analysis of mapmodulin revealed four consecutive stretches of homology between the carboxyl-terminal region of CLIP-170 and the amino-terminal region of mapmodulin. Pierre *et al.* (9) found that the carboxyl-terminal domain appears to be involved in anchoring the protein to patches at the plasma membrane, while the conserved repeated motif present in the amino-terminal domain of CLIP-170 regulates its binding to microtubules *in vivo*. Future experiments will be needed to determine whether the CLIP-170-related portions of mapmodulin mediate membrane association.

Despite sequence similarity with CLIP-170, mapmodulin does not appear to represent the key cytosolic factor responsible for direct Golgi binding to microtubules. Mapmodulin failed to enhance significantly the association of KCl-treated Golgi membranes with microtubules using a microtubule co-sedimentation assay. Under identical conditions, total CHO cytosol strongly stimulated microtubule co-sedimentation. A similar assay enabled Kreis and co-workers (8) to show that CLIP-170 linked endocytic carrier vesicles to microtubules.

What is the significance of the identity of mapmodulin with PHAPI? At low ionic strength (10 mM PIPES, pH 6.6, 40 mM NaCl), PHAPI bound to the peptide KGLRKSNAEERRGPL but not to the peptide KAARKAAAAERRAAA; PHAPI was released by increasing the NaCl concentration above 160 mM (3). Because of the charge similarity between the HLA-DR2 $\alpha$  peptide and the corresponding control peptide, the authors concluded that the interaction showed sequence specificity beyond the general charge distribution of the lysine and arginine residues (3). Although we cannot explain the physiological significance of PHAPI (mapmodulin) binding to the HLA-DR2 $\alpha$  peptide, it is interesting to note that MAP proteins utilize a basic domain for interaction with microtubules (reviewed in Ref. 13). An example of this tubulin-binding sequence, which is repeated in MAP2 protein, KCGSLKNIRHRPGGG, shares remarkable compositional similarity with the test sequence employed to isolate PHAPI. The overall spacing of basic residues and the presence and relative distribution of the specific, bulky amino acids leucine, asparagine, and proline are strikingly conserved between the HLA-DR2 $\alpha$  peptide and the tubulin binding domain of MAP2.

Mapmodulin/PHAPI is likely the human homolog of a protein described as rat "leucine-rich acidic nuclear protein" (LANP; 83.6% amino acid identity; Refs. 4 and 14). LANP was reported to be localized to the nuclei and cytoplasm of Purkinje cells. Our immunofluorescence analysis using affinity-purified antibodies and a variety of cell types did not reveal significant quantities of mapmodulin in the nucleus; mapmodulin was predominantly in the cytosol and on Golgi and ER membranes. It remains possible that our antibody recognizes a different phos-

phorylated form of mapmodulin than that studied in this report.

Chen *et al.* (5) have recently reported the cloning of cDNAs encoding the human and murine PHAPI, which they named pp32. This protein was previously identified as a phosphoprotein that is highly expressed in cancer tissues and self-renewing cell populations. Our data confirm the conclusion that PHAPI is a phosphoprotein in CHO cells.

Li *et al.* (6, 7) purified and cloned a potent and specific protein phosphatase 2A inhibitor,  $I_1^{PP2A}$ , which they later found to be identical to PHAPI. In the present study, we confirmed that commercial  $I_1^{PP2A}$  is a potent phosphatase inhibitor. However, we were unable to detect any phosphatase inhibitory activity in preparations of either native or recombinant mapmodulin. It is important to note that the purification procedure of both bovine kidney and *E. coli*-purified  $I_1^{PP2A}$  includes poly(L-lysine)-agarose and elution with high salt (6, 7). Moreover,  $I_1^{PP2A}$  was reported to elute from a gel filtration column as a  $\geq 250$  kDa form, rather than the native  $\sim 90$  kDa trimeric form of mapmodulin/PHAPI (this study). Li *et al.* (6, 7) stated that differences in protein phosphatase 2A inhibitor activity in the extracts from control and isopropyl-1-thio- $\beta$ -D-galactopyranoside-treated cells could not be distinguished prior to chromatography on poly(L-lysine)-agarose. These results imply that  $I_1^{PP2A}$  may be active in an aggregated form. We have shown that the native form of PHAPI/mapmodulin is not active as a phosphatase inhibitor. However, it remains entirely plausible that under certain conditions in living cells, the potential of mapmodulin to act as a phosphatase inhibitor is in some way unmasked.

We discovered mapmodulin based on its ability to stimulate the microtubule-dependent and cytoplasmic dynein-dependent localization of Golgi complexes in eukaryotic cells. This protein has a number of interesting biochemical features, including regulation by phosphorylation-induced conformational changes and MAP-binding capacity. A future challenge will be to elucidate further the biological roles of the mapmodulin/PHAPI phosphoprotein in mammalian cells.

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